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RESEARCH ARTICLE

The Effects of Thyme Oil and Thymol on Hepatic Gene Expression Levels in Rabbits with High Cholesterol Diet-Induced Hepatic Lipidosis

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ABSTRACT

Rabbits fed a high cholesterol diet (HCD) exhibit various physiopathologic features of hepatic lipidosis. This study aimed to investigate the effects of thyme oil (TO) and thymol (T) on inflammation-related hepatic gene expression in a rabbit model of high cholesterol diet–induced hepatic lipidosis. Male New Zealand rabbits were divided into six groups. The groups were: Standard rabbit diet (SD, n= 8), Standard rabbit diet + thymol (SD+T, n= 8), Standard rabbit diet + thyme oil (SD+TO, n= 8), High cholesterol diet (HCD, n=8), High cholesterol diet + thymol (HCD+T, n= 8), and High cholesterol diet + thyme oil (HCD+TO, n= 8). Blood samples were collected at weeks 0, 4, 8, and 11 of the study. Total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglyceride (TG) levels were analyzed. The mRNA expression levels of inflammation-related genes from liver tissue were analyzed by the real-time polymerase chain reaction (RT-PCR) method. The expression levels of *interleukin 4* (*IL4*), *IL17* and *interferon gamma* (*IFNγ*) genes were lower, whereas the expression levels of *IL9*, *IL13*, *IL18* and *RAR-related orphan receptor gamma* (*RORγ*) genes were higher in rabbits fed with HCD compared to the normal diet group ($p < 0.05$). Thymol increased *T-box transcription factor* (*Tbet*), *IL4*, *IL17A,* and *IL18* gene expression. Thyme oil increased *GATA-binding protein 3* (*Gata3*)and *IL18* gene expression. In conclusion, an HCD successfully established a non-obese rabbit model of hepatic lipidosis characterized by microvesicular steatosis, liver injury, and immune gene alterations. Thyme oil and thymol modulated immune responses by affecting key cytokine expressions but did not improve lipid profiles or liver histopathology. These results suggest that the immunomodulatory effects of thyme compounds are complex and their therapeutic potential may depend on specific disease contexts and treatment parameters. Further research is needed to clarify their role in hepatic lipidosis management.

Keywords: Hepatic lipidosis, High cholesterol diet, Hypercholesterolemia, Thyme oil, Thymol

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Yüksek Kolesterol Diyetiyle İndüklenmiş Hepatik Lipidozisli Tavşanlarda Kekik Yağı ve Timolün Karaciğer Gen Ekspresyonu Düzeylerine Etkisi

ÖZ

Yüksek kolesterol diyetiyle beslenen tavşanlar hepatik lipidozisin çeşitli fizyopatolojik özelliklerini gösterirler. Bu çalışmada, yüksek kolestrol diyeti ile indüklenmiş non-obez hepatik lipidozis tavşan modelinde, kekik yağı (TO) ve timol'ün (T) inflamasyonla ilişkili hepatik gen ekspresyon seviyeleri üzerine etkisinin araştırılması amaçlandı. Erkek Yeni Zelanda ırkı tavşanlar altı gruba ayrıldı: Standart tavşan yemi (SD, n= 8), Standart tavşan yemi + timol (SD+T, n= 8), Standart tavşan yemi + kekik yağı (SD+TO, n=8), Yüksek kolesterol diyeti (HCD, n= 8), Yüksek kolesterol diyeti + timol (HCD+T, n= 8) ve Yüksek kolesterol diyeti + kekik yağı (HCD+TO, n= 8). Çalışma süresince 0., 4., 8. ve 11. haftalarda kan örnekleri alındı. Total kolesterol (TC), yüksek yoğunluklu lipoprotein kolesterol (HDL-C), düşük yoğunluklu lipoprotein kolesterol (LDL-C) ve trigliserit (TG) düzeyleri analiz edildi. Karaciğer dokusundan inflamasyonla ilişkili genlerin mRNA ekspresyon seviyeleri gerçek zamanlı polimeraz zincir reaksiyonu (RT-PCR) yöntemiyle analiz edildi. Yüksek kolesterol diyetiyle beslenen tavşanlarda, normal diyet grubuna kıyasla *interlökin 4* (*IL4*), *IL17A* ve *interferon gamma* (*IFNγ*) geninin ekspresyon seviyesi düşük, *IL9*, *IL13*, *IL18* ve *RAR-related orphan receptor gamma* (*RORγ*) genlerinin ekspresyon seviyesinin yüksek olduğu belirlendi ($p< 0.05$). Timol *T-box transkripsiyon faktörü* (*Tbet*), *IL4*, *IL17A* ve *IL18* gen ekspresyonunu artırmıştır. Kekik yağı *GATA bağlayıcı protein 3* (*Gata3*) ve *IL18* gen ekspresyonunu artırmıştır. Sonuç olarak, yüksek kolesterol içeren diyet, mikrovakuoler steatoz, karaciğer hasarı ve immün gen ifadelerinde değişikliklerle karakterize edilen, obez olmayan bir tavşan hepatik lipidozis modeli başarıyla oluşturmuştur. Kekik yağı ve timol, önemli sitokin ekspresyonlarını etkileyerek bağışıklık yanıtlarını modüle etmiş, ancak lipid profilleri veya karaciğer histopatolojisini iyileştirememiştir. Bu sonuçlar, kekik bileşiklerinin immün modülatör etkilerinin karmaşık olduğunu ve terapötik potansiyellerinin hastalığın özel bağlamı ve tedavi parametrelerine bağlı olabileceğini göstermektedir. Hepatik lipidozis yönetimindeki rollerini netleştirmek için daha fazla araştırmaya ihtiyaç vardır.

Keywords: Hepatik lipidozis, Hiperkolesterolemi, Kekik yağı, Timol, Yüksek kolesterol diyeti

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INTRODUCTION

Epidemiological studies show that a high cholesterol diet (HCD) is linked to hepatic lipidosis (Kainuma et al. 2006). Rabbits fed an HCD show various physiopathological features of hepatic lipidosis (Kim et al. 2014; Kim et al. 2017). The hypercholesterolaemic rabbit model is also widely used in atherosclerosis studies due to their high sensitivity to dietary cholesterol and rapid development of atherosclerosis (Lozano et al. 2019). Since this model does not show insulin resistance or obesity, it is mainly used in studies of non-alcoholic fatty liver disease (NAFLD) related to hyperlipidaemia (Kainuma et al. 2006). In rabbits, fatty liver is induced by feeding a HCD (1% w/w) for two weeks or a low cholesterol diet (LCD) (0.3% w/w) for 16 weeks, and the livers of these rabbits show typical pathological features of hepatosteatosis (Kainuma et al. 2006; Kim et al. 2017). Cholesterol overload stimulates de novo lipogenesis by increasing liver X receptor-α (LXRα) expression and activates fatty acid synthesis by increasing the level of oxysterol, a metabolite in the sterol regulatory element binding protein-1c (SREBP-1c) pathway (Kainuma et al. 2006; Lozano et al. 2019). Inflammatory stress also exacerbates hepatic cholesterol accumulation by impairing cellular cholesterol export (Kainuma et al. 2006; Kim et al. 2017; Lozano et al. 2019).

Thyme (*Thymus vulgaris* [*T. vulgaris*]) is widely recognized as a therapeutic plant due to its biological and pharmacological qualities (Prasanth Reddy et al. 2014). In traditional medicine, leaves and flowering portions of Thymus species are commonly used as a tonic and herbal tea, antiseptic, antitussive, and carminative, as well as for treating colds (Prasanth Reddy et al. 2014). Thyme oils (TO) and extracts are widely utilized in the medicinal, cosmetic, and perfume industries, as well as for flavoring and preserving a variety of foods (Prasanth Reddy et al. 2014). Thymol (isopropyl-5-methylphenol) is a natural monoterpene phenol obtained from thyme species (Marchese et al. 2016). Thyme and thymol, one of its active components, have antioxidant, anti-inflammatory, anti-bacterial, anti-fungal, anti-cancer activity, hepatoprotective properties, immunomodulatory and platelet aggregation inhibitor properties (Rašković et al. 2015; Marchese et al. 2016; El Boshy et al. 2019; Lee et al. 2023; Sheng et al. 2024). It has been stated that thyme may be a promising natural therapeutic drug in improving intestinal conditions associated with obesity and high-fat diet (Lee et al. 2023). Thyme polyphenol-rich extract substantially reduces high-fat diet-induced NAFLD via regulating the gut-liver axis, focusing on gut microbiota and bile acid metabolism (Sheng et al. 2024). Thyme extract reduced lead-induced stress in hepatic and renal tissues and showed promise as an immunomodulator, antioxidant, and protective agent against lead toxicity (El Boshy et al. 2019). Thyme preparations exerted antioxidant effects in the liver by preventing carbon tetrachloride-induced increase of lipid peroxidation (Rašković et al. 2015).

Although the aetiology of NAFLD is multifactorial and remains largely enigmatic, it is well established that inflammation is a central component of NAFLD pathogenesis. Inflammation disrupted PPAR-LXR-CYP7A1/ABCA1-mediated bile acid production and cholesterol efflux, leading to increased cholesterol buildup in the livers and HepG2 cells (Chen et al. 2012). In addition, excessive cholesterol accumulation in cells may exacerbate NAFLD by inducing endoplasmic reticulum (ER) stress, oxidative stress, and apoptosis (Senokuchi et al. 2008). Thyme extract may suppress TNF-α, IL-6, and other inflammatory cytokines. It has the potential to protect against disease-related consequences and be used as a therapeutic agent (Nadi et al. 2023). Thyme extract significantly suppressed the synthesis and gene expression of inflammatory mediators, whereas it upregulated both synthesis and gene expression of the anti-inflammatory cytokine IL-10 (Ocaña et al. 2012). Thyme extract acts as an anti-inflammatory drug by scavenging nitric oxide radicals that contribute to the beginning of inflammatory conditions and greatly inhibiting the production of inducible nitric oxide synthase mRNA (Vigo et al. 2004). Thyme polyphenol-rich extract improves intestinal barrier function and reduces inflammation by increasing tight junction protein expression (ZO-1 and occluding) and inhibiting the TLR4/NF-κB pathway in high-fat diet-fed rats (Sheng et al. 2023). The roles of critical immune mediators, particularly inflammation-related genes, as well as loci of immune activation, immune signalling pathways, and mechanisms underlying disease progression, are still poorly understood. Knowledge of genes associated with inflammation in the liver, particularly in a high-cholesterol diet–induced hepatic lipidosis model, may help to identify and develop new therapeutic targets. Therefore, this study aimed to investigate the effects of thyme oil and thymol on inflammation-related hepatic gene expression in a rabbit model of high cholesterol diet-induced hepatic lipidosis.

MATERIAL and METHOD

Ethics Committee

Ethics committee approval was received for this study from the Animal Experiments Local Ethics Committee of Erciyes University (No: 20/069).

Animals

A total of 48 healthy male New Zealand rabbits with an average weight of 3.41±0.56 kg and 10-12 weeks of age were used in the study. The rabbits were housed in single 60x60x30 cm rabbit cages at Erciyes

University Experimental Research and Application Center (DEKAM) during the 15-day adaptation period and throughout the study. During the study, the daily diet of each animal was restricted to 100 g to maintain consistent final body weights across all groups. Water was provided ad libitum. Rabbits were kept in single cages with appropriate ventilation, standardized light (12 hours light/12 hours darkness daily), and temperature (22±1 ºC) conditions throughout the experiment.

After a 2-week adaptation period, the rabbits were randomly divided into 2 groups: standard diet (SD) and high cholesterol diet (HCD). The rabbits in the SD groups were fed a standard rabbit diet (Optima Besin Maddeleri San. ve Tic. A.Ş, Lüleburgaz/Kırklareli). Rabbits in the high cholesterol diet (HCD) groups were fed a standard rabbit dietsupplemented with 1.0% (wt/wt) cholesterol. These groups (SD, HCD) were then divided into 3 subgroups (8 rabbits/group). The standard diet (SD) group was fed only standard rabbit chow for 11 weeks; standard diet (SD) + thymol (SD+T) group, standard rabbit diet + thymol (6 mg/kg, oral) for 11 weeks; standard diet + thyme oil (SD+TO) group, standard rabbit diet + thyme oil (0.1mL, oral) for 11 weeks); high cholesterol diet group (HCD), fed standard rabbit chow containing 1% (wt/wt) cholesterol [≥92. 5% (GC), Sigma-Aldrich]; high cholesterol diet + thymol group (HCD+T), fed standard rabbit chow containing 1% cholesterol + thymol (6 mg/kg, oral) for 11 weeks; high cholesterol diet + thyme oil group (HCD+TO), fed standard rabbit chow containing 1% cholesterol + thyme oil (0.1mL, oral) for 11 weeks.

Measuring Body Weights

The body weights of all rabbits were recorded using a digital weighing scale (PLUSMED pM-BS01) at weeks 0, 4, 8, and 11 during the study.

Blood Sample Collection

A 3.5 mL blood sample was collected from the marginal ear vein of rabbits into blue-capped vacuum tubes containing 3.2% (0.109 M) sodium citrate (BD Vacutainer®, Becton Dickinson, USA). Additionally, 2 mL of blood was drawn from the same vein into yellow-capped serum separator tubes (BD Vacutainer®, Becton Dickinson, USA). Blood samples were centrifuged at 3000 × g for 15 minutes (ROTOFIX 32A, Hettich, Germany), and the resulting serum and plasma were aliquoted into 0.5 mL portions and stored at -20°C under appropriate conditions.

Analyses of Lipid Parameters

Analyses of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides (TG) were performed by the Gülser-Dr. Mustafa Gündoğdu Central Laboratory at Erciyes University, through service procurement.

Gene Expression Analyses

At the end of the experiment (week 11), following overnight fasting, all rabbits were euthanized under anesthesia with pentobarbital sodium (50 mg/kg). Liver tissue samples of euthanized rabbits were stored at -80°C in Eppendorf tubes containing 300 µL TRIzol reagent (Qiagen, Germany) for mRNA isolation. Total RNA isolation, real-time PCR, and gene expression analysis of liver tissue samples were performed at Erciyes University, Betül-Ziya Eren Genome and Stem Cell Research Center.

Total RNA Isolation and Determination of RNA Concentration

mRNA isolation was performed according to the total RNA extraction procedure with Qiazol (Qiagen, Germany). After thawing, the tissues were disintegrated with a homogenizer (Scilogex D-160, Malaysia). 200 µL of chloroform (Merck, Germany) was added. Centrifuged at 12.000 g for 15 min at +4 ºC. The supernatant was transferred to a new tube. 200 µL isopropanol (Merck, Germany) (1:1 ratio) was added. Incubated for 10 min at room temperature. Centrifuged at 12.000 g for 10 min at +4 ºC. The supernatant was removed. 1000 µL of ethanol was added and centrifuged at 7500 g for 5 min at +4 ºC. The supernatant was removed and allowed to dry for 10 min. 50 µL of nuclease-free water was added and dissolved. RNA measurements were performed with a nanodrop spectrophotometer.

Complementary-DNA Synthesis

Complementary DNA (cDNA) synthesis was performed according to the protocol of the high-Capacity cDNA reverse transcription kit (Thermo Fisher Scientific, USA). Mixture preparation: At this stage, the following amounts of mixture were prepared for one sample in a 0.2 µl Eppendorf tube. To this mixture, 500 ng/µl of isolated RNA was added and incubated in a thermal cycler (Qiagen, Germany). cDNA reverse transcription reaction composition: Total reaction (20 µL) = 10× RT Buffer (2.0 µL) + 25× dNTP Mix (100 mM) (0.8 µL) + 10× RT Random Primers (2.0 µL) + MultiScribe™ Reverse Transcriptase (1.0 µL) + Nuclease-free H2O (4.2 µL) + RNA (10 µL). Thermal Cycler Program: Step 1 (Temp.: 25 °C, Time: 10 sec), Step 2 (Temp.: 37 °C, Time:120 sec), Step 3 (Temp.: 85 °C, Time: 5 sec), Step 4 (Temp.: 4 °C, Time: ∞).

Real Time PCR Analysis

The Real-Time expression phase of the study was performed using a Light Cycler Nano (Roche Ltd., Mannheim, Germany) Real-Time PCR device and SYBR Green PCR Master Mix (Thermo Fisher Scientific). DNA synthesized samples were placed on a block at +4 °C 10 minutes before the study and

kept for a while. The *Hprt1* gene was used as a housekeeping gene in the study. Two separate working mixtures were prepared for the target and control genes to be studied. SYBR Green Master Mix, Primer F, Primer R, PCR Grade H_2O, and c-DNA were taken in 96-well plates in the following amounts for one sample. Samples with Light Cycler Nano Real-Time PCR device (Roche, Switzerland): 10 minutes at 95 °C, 10 seconds at 95 °C, 10 seconds at 60 °C, 10 seconds at 72 °C, 45 cycles of 10 seconds at 95 °C and 30 seconds at 95 °C. The melting temperature was analyzed by increasing the amount of heat by 0.1 °C per second at temperatures between 65 °C and 95 °C, and a melting graph analysis of the amount of fluorescence generated over time. Gene data for each sample were normalized to the *Hprt1* gene.

The Ct averages of *signal transducer and activator of transcription 4* (*STAT4*), *interferon gamma* (*IFNγ*), *Foxp3* (*Forkhead box P3*), *T-box transcription factor* (*Tbet*), *Gata3* (*GATA binding protein 3*), *granulocyte-macrophage colony-stimulating factor* (*GMCSF*), *RORγ* (*RAR-related orphan receptor gamma*), *interleukin 4* (*IL4*), *IL5*, *IL8*, *IL9*, *IL10*, *IL13*, *IL17A*, *IL18* genes were subtracted from the Ct averages of Hprt1 gene (Delta Ct: Ct [Control Gene]-Ct [Target Gene]) and the data were normalized. Power was calculated with the formula [Power (2ΔCt)], and the Log10 of the power values was taken and made suitable for statistical study. Arbitrary relative expression units were calculated by the division of the expression of the gene of interest by *hypoxanthine phosphoribosyltransferase 1* (*Hprt1*) mRNA expression. Primer sequences for each gen are given in Table 1.

Table 1. Primer sequences(http://www.ncbi.nlm.nih.gov)

Gene	***5′ to 3'***
STAT4	F: 5'-CAGATCATACAGCCAATGTGC-3' R: 5'-GGTTGAGGTTTTTGCGGAGT-3'
IFNγ	F: 5'-TGCCAGGACACACTAACCAGAG-3' R: 5'-TGTCACTCTCCTCTTTCCAATTCC-3'
Tbet	F: 5'-CCTTCCAAGAGACGCAGTTC-3' R: 5'-AGGAAGCTCGGGGTAGAAAC-3'
IL4	F: 5'-CGACATCATCCTACCCGAAGTC-3' R: 5'-CCTCTCTCTCGGTTGTGTTCTTG-3'
IL5	F: 5'-AGACCCTGACACTGCTCTCA-3' R: 5'-AGGTGATGATTTTTATGGACCGGA-3'
IL13	F: 5'-TCATCGAGGAGCTGGTCAAC-3' R: 5'-AGCCTTGTCTGTGCAGAGTC-3'
Gata3	F: 5'-AGGCAGGGAGTGTGTGAACT-3' R: 5'-CGTCGTGGTCTGACAGTTTG-3'
IL17A	F: 5'-CCAGCAAGAGATCCTGGTCCTA-3' R: 5'-ATGGATGATGGGGGTTACACAG-3'
GMCSF	F: 5'-TTCCTCCTAGGCAGTGTGGT-3' R: 5'-TCTACCATTTCGCCCAGCAC-3'
***RORγ* (*RORC*)**	F:5'-GGGCTTCATACCACCTTGAA-3' R: 5'-GTGCTCTGGGCCTATCTCTG-3'
IL9	F: 5'-ATCCCGTCTGACAACTGCAC3' R: 5'-GGCTTCCACCGTTCTTCTCA3'
IL10	F: 5'-CTTTGGCAGGGTGAAGACTTTC-3' R: 5'-AACTGGATCATCTCCGACAAGG-3'
IL18	F: 5'-ACCAAGGACAGCAACCTGTGTT-3' R: 5'-ACAGAGAGGCTTACAGCCATGC-3'
Foxp3	F: 5'-CACAGTGCCCCTAGTCATGG-3' R: 5'-CTGAGAGCTGGTGCATGAAGT-3'
IL8	F: 5'-CCACACCTTTCCATCCCAAAT-3' R: 5'-CTTCTGCACCCACTTTTCCTTG-3'
Hprt1	F:5'-GCAGACCTTGCTTTCCTTGGT-3' R: 5'-GCAGGCTTGCGACCTTGAC-3'

IFNγ: *Interferon gamma*, ***IL8***: *Interleukin 8*, ***Foxp3***: *Forkhead box P3*, ***IL18***: *Interleukin 18*, ***IL10***: *Interleukin 10*, **IL9**: *Interleukin 9*, ***RORγ*(*RORC*)**: *RAR-related orphan receptor gamma*, ***GMCSF***: *Granulocyte-macrophage colony-stimulating factor*, ***IL17A***: *Interleukin 17A*, ***Gata3***: *GATA binding protein 3*, ***IL-13***: *Interleukin 13*, ***IL4***: *Interleukin 4*, ***Tbet***: *T-box transcription factor*, ***STAT4***: *Signal transducer and activator of transcription 4*, ***Hprt*1**: *Hypoxanthine phosphoribosyltransferase*, **F**: Forward, **R**: Reverse

Histopathological Analyses

Histopathologic examinations were performed at the Department of Pathology, Erciyes University Faculty of Medicine Health Application and Research Center, Kayseri, Türkiye. The liver was dissected directly under physiologic saline perfusion, and the right lobe was fixed in 10% formalin solution for 1 hour. After the tissues were processed using the routine method, 3-5 µm thick sections were taken and stained with hematoxylin-eosin (H&E) and examined under a light microscope.

Statistical Analysis

Statistical analyses were performed using SPSS for Windows Release 25.0 (SPSS Inc., Chicago, IL, USA). All data were analysed graphically and tested for normality using the Shapiro-Wilk test and the Q-Q plot. All data that passed the normality test were expressed as mean and standard deviation (SD). One-way ANOVA (alternative: Kruskal-Wallis Test) was used for comparisons between groups. Repeated Measures ANOVA was used for comparisons between times. Bonferroni and Tukey tests were used for multiple comparisons. Graphs were drawn with the free version of GraphPad Prism 9.0 software (GraphPad Software Inc., San Diego, CA, USA). A p-value of < 0.05 was considered statistically significant.

RESULTS

Body Weight Findings

Body weight of rabbits in the SD groups (SD, SD+T, and SD+TO) increased. On the other hand, the body weights of rabbits in the HCD groups (HCD, HCD+T, HCD+TO) decreased slightly, and this phenomenon persisted until the end of the experiment. A similar effect was observed in the SD+TO group, but this effect disappeared after the 4th week. In the 11th week, rabbits in the HCD groups had a lower mean body weight compared to those fed an SD group ($p< 0.05$) (Figure 1).

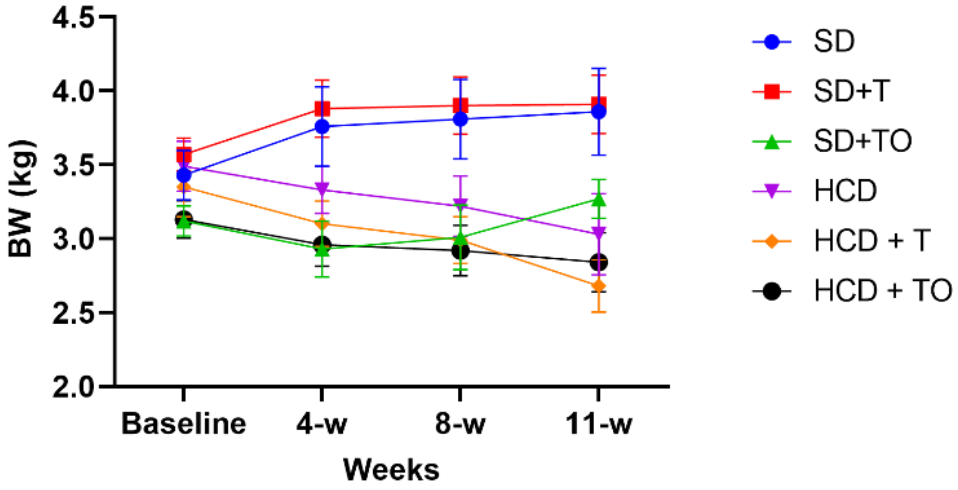

Figure 1: Changes in body weight according to time in SD and HCD groups, BW; body weight, SD (n = 8); standard diet group, SD+T (n = 8); standard diet + thymol group, SD+TO (n = 8); standard diet + thyme oil group, HCD (n = 8); high cholesterol diet group, HCD+T (n = 8); high cholesterol diet + thymol group, HCD+TO (n = 8); high cholesterol diet + thyme oil group.

At week 4, the mean body weight of rabbits in the ND-TO group (2.93 ± 0.50) was significantly lower than in the ND (3.76 ± 0.66, p = 0.048) and ND+T (3.89 ± 0.55, p = 0.008) groups. The HCD-T (3.10 ± 0.41, p = 0.043) and HCD-TO (2.96 ± 0.36, p = 0.016) groups also had lower body weights than the ND+T group (3.89 ± 0.55) at week 4.

At week 8, body weight in the ND-T group (3.90 ± 0.55) was higher than in the ND-TO (3.01 ± 0.58, p= 0.031), HCD+T (2.99 ± 0.42, p= 0.027), and HCD+TO (2.92 ± 0.42, p= 0.019) groups.

At week 11, rabbits in the ND (3.86 ± 0.72) and ND+T (3.91 ± 0.56) groups had higher body weights than those in the HCD+T (2.68 ± 0.47, p= 0.008 and p= 0.002, respectively) and HCD+TO (2.84 ± 0.49, p= 0.039 and p= 0.016, respectively) groups.

No significant changes over time were observed in ND, ND+T, ND-TO, and HCD groups. However, in the HCD+T group, body weight at weeks 0 (3.35 ± 0.20) and 4 (3.10 ± 0.16) was higher than at week 11 (2.68 ± 0.18) (p= 0.004 and p= 0.049). Similarly, in the HCD+TO group, body weight at week 0 (3.13 ± 0.31) was higher than at weeks 4 (2.96 ± 0.36), 8 (2.92 ± 0.42), and 11 (2.84 ± 0.49) (p= 0.011, p= 0.013, p = 0.022).

Lipid Profile Findings

The high cholesterol diet groups (HCD, HCD+T, and HCD+TO) had significantly higher TC, TG, LDL-C, and HDL-C. The mean triglyceride concentration of rabbits in the HCD (210.86 ± 13.63 mg/dl) and HCD + TO (151.50 ± 34.57 mg/dl) groups at week 4 was higher than that of the SD + TO (67.29 ± 9.62 mg/dl) group ($p< 0.05$). The mean triglyceride concentration of rabbits in HCD + T (216.00 ± 20.13 mg/dl) and HCD + TO (294.33 ± 92.27 mg/dl) groups at week 8 was higher than SD (139.67 ± 22.21 mg/dl), SD+T (102.0 ± 17.10 mg/dl), SD-TO (124.71 ± 10.14 mg/dl) and HCD (142.86 ± 73.66 mg/dl) groups ($p< 0.05$). The mean triglyceride concentration of rabbits in HCD (344.43 ± 39.58 mg/dl), HCD + T (389.71 ± 21.20 mg/dl) and HCD + TO (405.33 ± 50.92 mg/dl) groups at week 11 was higher than SD (228.0 ± 35.63 mg/dl), SD+T (178.50 ± 35.81 mg/dl) and SD-TO (248.29 ± 28.33 mg/dl) groups ($p<0.05$) (Figure 2).

The mean triglyceride concentration of the SD group at week 11 (228.0 ± 35.63 mg/dl) was higher than at week 0 (114.50 ± 32.15 mg/dl) ($p= 0.001$). The mean triglyceride concentration of the SD+T group at week 11 (178.50 ± 35.81 mg/dl) was higher than that at week 0 (145.50 ± 19.78 mg/dl), week 4 (129.0 ± 5.35 mg/dl) and week 8 (102.0 ± 17.10 mg/dl) ($p<0.05$) (Figure 2).

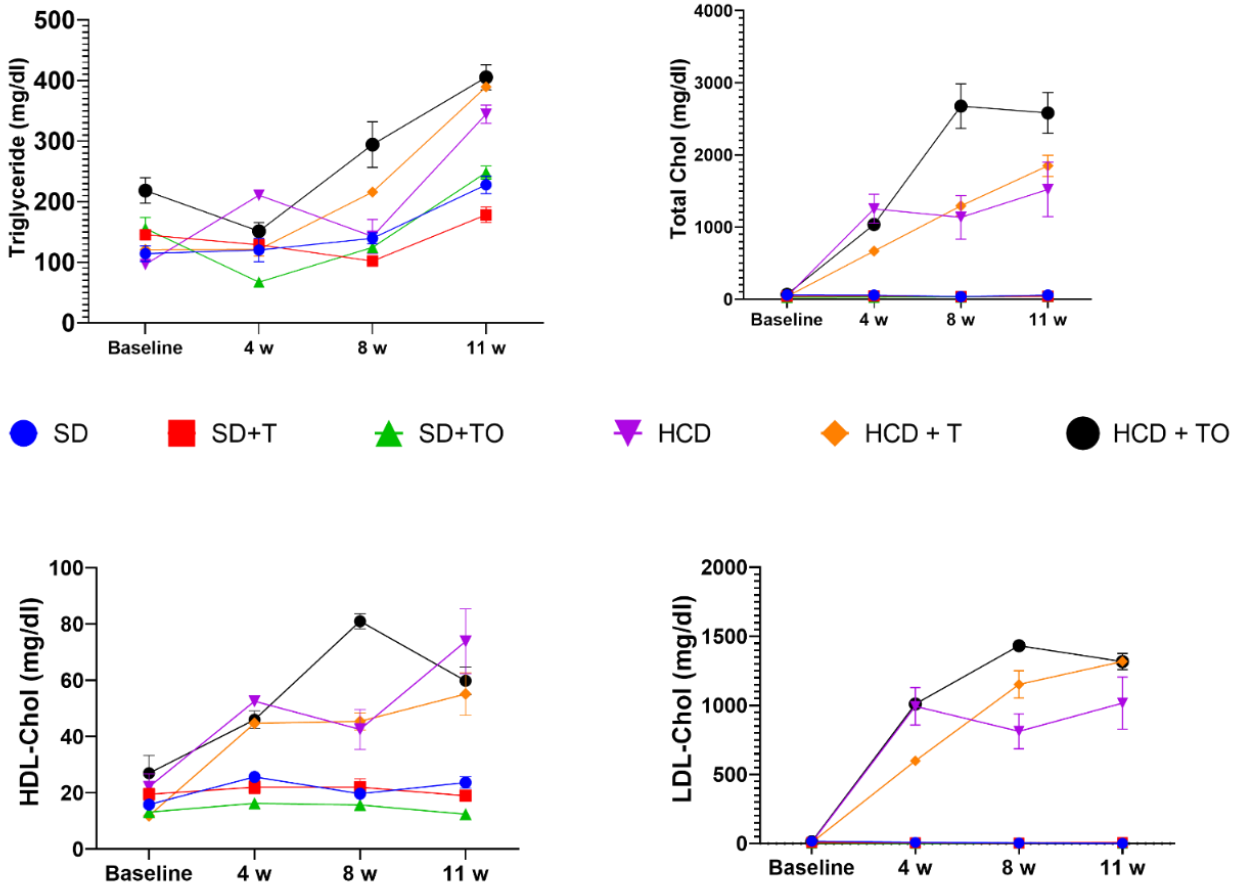

Figure 2: Changes in lipid concentrations of rabbits in SD and HCD groups according to time, BW; body weight, SD; standard diet group, SD+T; standard diet + thymol group, SD+TO; standard diet + thyme oil, HCD; high cholesterol diet, HCD+T; high cholesterol diet + thymol, HCD+TO; high cholesterol diet + thyme oil.

Hepatic Gene Expression Findings

The mRNA expression levels of inflammation-related genes in liver tissue samples are shown in Figure 3-4. There was a statistical difference between the groups in terms of the expression level of *IFNγ* ($p < 0.001$). The relative gene expression levels of *IFNγ* in SD + T, SD + TO, HCD, HCD + T, and HCD + TO groups were significantly higher than those of the SD group. There was a statistical difference between the groups in terms of the expression level of *IL4* ($p= 0.006$). The relative gene expression level of *IL4* in the SD + T group was significantly higher than that of the HCD and HCD + TO groups. There was a statistical difference between the groups in terms of the expression level of *Tbet* ($p < 0.001$). The relative gene expression level of *Tbet* in SD and SD+T groups

was significantly lower than that in SD+TO group, while the relative gene expression level in SD+TO group was significantly higher than that in HCD and HCD+TO groups. There was a statistical difference between the groups in terms of the expression level of *Gata3* ($p < 0.001$). The relative gene expression level of *Gata3* in the SD group was significantly lower than that of the SD + T and SD + TO groups. The relative gene expression level of *Gata3* in the SD+T group was significantly lower than in the SD+TO group and significantly higher than in the HCD and HCD+T groups. The relative gene expression level of *Gata3* in the SD+TO group was significantly lower than that of the SD+TO group and significantly higher than that of the HCD, HCD+T, and HCD+TO groups. There was a statistical difference between the groups in terms of the expression level of *IL17A* ($p= 0.002$). The relative gene expression level of *IL17A* in the SD group was significantly lower than that in the SD+T group, while the relative gene expression level of IL17A in the SD+T group was significantly higher than that in the HCD, HCD+T, and HCD+TO groups. There was a statistical difference between the groups in terms of the expression level of *IL18* ($p= 0.041$). The relative gene expression level of *IL18* in the HCD + T group was significantly higher than that in the SD group. There was a statistical difference between the groups in terms of the expression level of *IL8* ($p= 0.016$). The relative gene expression level of *IL8* in the HCD+T group was significantly higher than that of the HCD and HCD+TO groups.

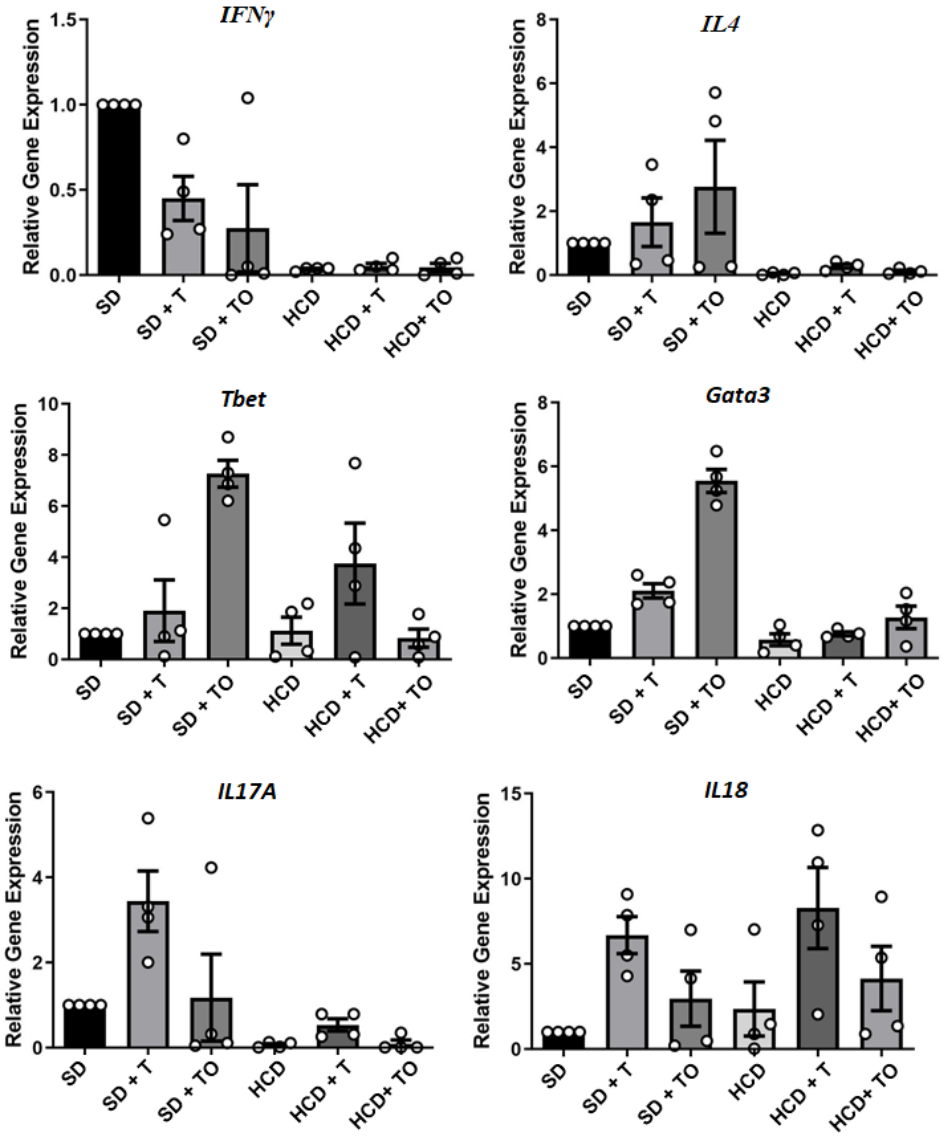

Figure 3: Comparison of hepatic gene expression levels of *IFNγ*, *Tbet*, *Gata3*, *IL4*, *IL18* and *IL17A*. *IFNγ*: *Interferon gamma*, *IL18*: *Interleukin 18*, *IL17A*: *Interleukin 17A*, *Gata3*: *GATA Binding Protein 3*, *IL4*: *Interleukin 4*, *Tbet*: *T-box transcription factor*.

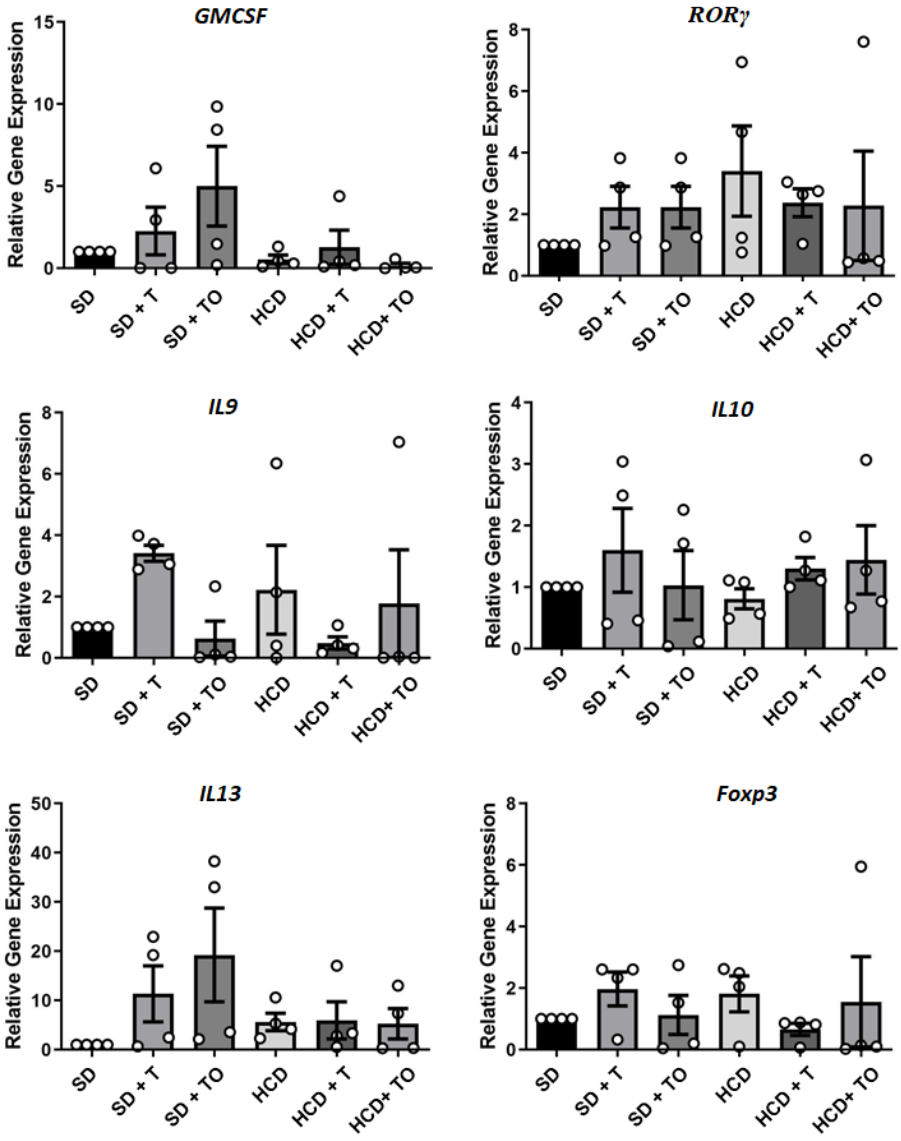

Figure 4: Comparison of hepatic gene expression levels of *Foxp3*, *IL9*, *IL10*, *RORγ*, *GMCSF,* and *IL13*. *Foxp3*: *Forkhead box P3*, *IL9*: *Interleukin 9*, *IL10*: *Interleukin 10*, *RORγ* (*RORC*): *RAR-related orphan receptor gamma*, *GMCSF*: *Granulocyte-macrophage colony-stimulating factor*, *IL13*: *Interleukin 13*.

Macroscopic and Histopathologic Findings

The livers of the rabbits in the SD-fed groups were macroscopically normal, and no lesions were observed. On the other hand, the livers of the rabbits fed with HCD were macroscopically observed to have yellowish discoloration, increased volume, blunted edges, shiny, slippery, and protruding cross-sectional surfaces, friable consistency, and some of them adhered to the diaphragm. In addition, ascites was observed in 1 of the rabbits in the HCD + T group. Histopathologic examination of the livers of the rabbits in the SD group showed normal liver structure. The histopathologic appearance of the livers of the rabbits in SD + T and SD + TO groups was the same with the histopathologic appearance of the livers of the rabbits in the SD group, no pathologic lesion was observed and they were found to have normal structure. Histopathologic appearance of the livers of HCD group animals showed balloon-like degeneration, fat vacuoles, hemosiderin pigment in hepatocytes, lymphocyte-rich mononuclear cell infiltrations in the portal areas, and an increase in the number of Kupffer cells. In the liver sections of rabbits in the HCD + T and HCD + TO groups, microscopically balloon-like degeneration and partially reduced fat vacuoles were observed. There was no change in the severity of hepatic findings in HCD + T and HCD + TO groups compared to the HCD group (Figures 5 and 6).

Figure 5: Macroscopic appearance of the livers of rabbits fed SD and HCD. SD; standard diet group, SD + T; standard diet + thymol group, SD + TO; standard diet + thyme oil, HCD; high cholesterol diet, HCD + T; high cholesterol diet + thymol, HCD + TO; high cholesterol diet + thyme oil.

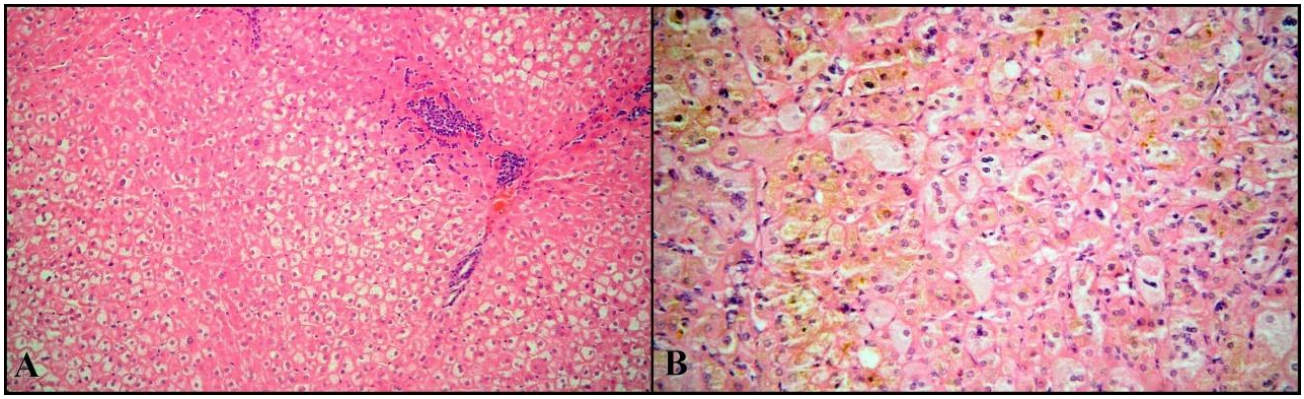

Figure 6: Histopathological appearance of the livers of the high cholesterol diet (HCD) group animals. (A) In the liver tissue of the high cholesterol diet (HCD) group, distinct morphological changes, intercellular irregularities, enlarged sinusoid areas, and local inflammatory foci were observed in hepatocytes. Disruption of the liver lobular structure and ballooning (swelling) of the hepatic cells are noted. (B) At higher magnification, dense fat accumulation (vacuole-like spaces), degeneration, areas of necrosis, and inflammatory cell infiltration are clearly observed in the hepatocyte cytoplasm. There is also marked steatosis in the liver and histopathological changes due to cell damage.

DISCUSSION

Cholesterol-fed rabbits have been widely used as an experimental model to investigate cardiovascular disorders associated with hypercholesterolemia and atherosclerosis (Kainuma et al., 2006; Kim et al., 2014; Kim and Kim, 2017). In addition to cardiovascular effects, a HCD has been shown to induce non-alcoholic fatty liver disease (NAFLD) in various studies (Kainuma et al., 2006; Kim et al., 2014). Notably, this diet appears to contribute more significantly to the development of non-obese NAFLD compared to the obese form of the disease (Kim et al., 2014; Kim and Kim, 2017). Consistent with these findings, in the present study, a non-obese hepatic lipidosis model characterized by hypertriglyceridemia, hypercholesterolemia, and fatty liver was successfully established in rabbits fed a high-cholesterol diet.

In the present study, consistent with the findings of Kim et al. (2014), blood levels of TC, TG, LDL-C, and HDL-C were significantly elevated in rabbits fed a high-cholesterol (1%) diet. Similarly, El Sebaei et al. (2019) reported increased serum levels of TC, triacylglycerol (TAG), and LDL-C in rabbits on a high-cholesterol diet compared to those fed a normal diet; however, no significant difference was observed in serum HDL-C levels. Cholesterol overload leads to

accumulation in the liver, and hepatic cholesterol accumulation is likely to contribute to the progression of NAFLD, analogous to the effects of hepatic triglyceride overload (Tous et al., 2005; Lozano et al., 2019). Our results demonstrated that treatment with thyme oil and thymol did not significantly affect TC, HDL-C, LDL-C, or TG levels.

In the present study, consistent with the findings of Kainuma et al. (2006), the livers of rabbits fed a high-cholesterol diet exhibited notable hepatocellular fat deposition, cellular ballooning, and mild infiltration of neutrophils. Although macrovesicular fatty changes were not clearly distinguishable, significant microvesicular fat accumulation and aggregation of enlarged hepatic stellate cells were observed, particularly in the perivenular regions. This pattern of microvesicular fat deposition has been emphasized by Fromenty and Pessayre (1995) as indicative of a more severe hepatic injury compared to macrovesicular fat, reflecting mitochondrial dysfunction and impaired β-oxidation pathways. Previous studies have demonstrated the hepatoprotective potential of thyme-derived compounds. For instance, Sheng et al. (2023) reported that supplementation with thyme polyphenol-rich extract alleviated high-fat diet-induced liver injury in C57 mice in a dose-dependent manner, resulting in improved liver histology and reduced oxidative stress markers. Similarly, Yan et al. (2021) showed that thyme ethanolic extract exerted protective effects in a laboratory model of alcoholic liver disease by decreasing inflammatory cytokines and enhancing antioxidant enzyme activity. Furthermore, Lahmi et al. (2023) observed that administration of thymol at a dose of 50 mg/kg in rats with fatty liver disease led to decreased serum liver enzyme levels, reduced histopathological degenerative changes, and restoration of normal liver architecture. Contrary to these reports, our study found that neither thyme oil nor thymol treatment significantly reduced the severity of fatty liver disease in rabbits fed a high-cholesterol diet when compared to control groups. This discrepancy may be attributed to differences in animal species, dosage, duration of treatment, or the specific hepatic insult model used. It is also possible that the pathophysiological mechanisms underlying cholesterol-induced hepatic lipidosis differ from those in alcoholic or other diet-induced liver injuries, which might influence the efficacy of thyme compounds. Further research is warranted to clarify the potential therapeutic roles of thyme oil and thymol in diverse models of hepatic steatosis and to determine optimal treatment parameters.

In the present study, both food intake and body weight of rabbits fed a high-cholesterol diet were reduced, and this trend persisted until the end of the experiment. At the conclusion of the study, rabbits in the high-cholesterol diet groups exhibited significantly lower mean body weights compared to those fed a normal diet ($p < 0.05$). Similarly, El Sebaei et al. (2019) reported that rabbits fed a hypercholesterolemic diet showed decreased body weight, daily feed intake, total weight gain, and feed conversion ratio at the end of the eighth week compared to rabbits on a normal diet. This reduction may be attributed to changes in the palatability of the pellet feed caused by the high cholesterol content. Another possible explanation involves endoplasmic reticulum (ER) stress and oxidative stress resulting from excessive cholesterol accumulation in hepatocytes (Senokuchi et al., 2008; Zhang et al., 2010).

In this study, the expression level of the *IFNγ* gene in rabbits fed an HCD was significantly lower than in the standard diet group ($p< 0.05$). Contrary to our findings, Inzaugarat et al. (2017) reported that the relative expression of the *IFNγ* gene in liver biopsy samples from patients with NAFLD was significantly higher compared to controls. The suppression of *IFNγ* gene expression observed in our HCD groups may be related to the non-obese nature of our model and the stress induced by elevated plasma cholesterol levels. Supporting this, Luo et al. (2013) demonstrated in a high-fat diet-induced steatohepatitis mouse model that fibrosis-related gene expressions (including *a-smooth muscle actin*, *type I collagen*, *matrix metalloproteinase-1 tissue inhibitor*, and *matrix metalloproteinase-2*) were significantly increased in wild-type mice but markedly suppressed in IFNγ-deficient knockout mice. Moreover, a human study by Ghaedi et al. (2021) found no significant effect of thyme supplementation on serum IFN-γ and TNF-α levels in individuals with NAFLD. In the present study, administration of thymol and thyme oil downregulated *IFNγ* gene expression in SD groups, which is thought to result from their anti-inflammatory properties, potentially exerting a protective effect on liver cells.

In the present study, the expression level of the *IL4* gene was significantly higher in the normal diet group compared to the HCD groups ($p < 0.05$), with strong gene suppression observed in the HCD groups. In patients with NAFLD, pro-inflammatory cytokines such as TNF-α and IL-6 are typically elevated, while levels of the anti-inflammatory cytokine IL-4 are decreased, and IL-10 levels remain unchanged (Das and Balakrishnan, 2011). Zhou et al. (2014) reported that thymol reduced ovalbumin-specific IgE levels, inhibited recruitment of inflammatory cells into the airways, and decreased IL-4, IL-5, and IL-13 levels in bronchoalveolar lavage fluid in a mouse model of asthma. In our study, treatment with thymol and thyme oil increased *IL4* gene expression in liver tissue. This upregulation is likely attributable to the anti-inflammatory properties of thymol and thyme oil. Although *IL4* gene expression was strongly suppressed in the high-cholesterol groups, thymol and thyme oil treatments effectively upregulated *IL4* expression, potentially contributing to the reduction of pathological inflammation.

In this study, thymol increased *T-bet* gene expression in rabbits fed both normal and HCD. Thyme oil upregulated *T-bet* expression in normal diet rabbits ($p< 0.05$) but downregulated it in the high-cholesterol group. While previous studies reported higher *T-bet* expression in NAFLD patients and HCD rabbits (Inzaugarat et al., 2017; Sheng et al., 2011), our study found no difference between the high-cholesterol and control groups. Notably, thymol treatment caused a 7-fold increase in *T-bet* expression, supporting its immunomodulatory role by promoting Th1 responses and inhibiting Th17-mediated inflammation. This is consistent with our IL-17 results, showing reduced IL-17 expression after thymol administration.

Zeyda et al. (2011) reported increased *Gata3* gene expression in the subcutaneous adipose tissue of obese patients compared to controls. In our study, an HCD suppressed hepatic *Gata3* gene expression. This suppression may be attributed to the non-obese nature of our model, as rabbits in the HCD group had lower body weights than those in the control groups. Treatment with thyme oil and thymol increased *Gata3* expression in rabbits fed both a normal diet and an HCD, with a greater increase observed in the thyme oil group. However, in the HCD groups, where gene suppression was pronounced, these treatments only slightly elevated *Gata3* expression. Notably, in parallel with the increase in *Gata3* expression, *IL4* and *IL13* genes were also upregulated in this study. Given that *Gata3* is known to promote the secretion of IL-4, IL-5, and IL-13 from Th2 cells (Yagi et al., 2011), these findings suggest that thyme oil and thymol may exert their anti-inflammatory effects, at least in part, through the activation of Th2-associated pathways.

Obese humans and animals exhibit elevated *IL17A* expression, associated with increased adipose tissue Th17 cell infiltration (Sumarac-Dumanovic et al., 2009). Anti-IL-17 antibodies have been shown to improve liver function, suppress Kupffer cell activation, and reduce pro-inflammatory cytokines via NF-$\varkappa$B inhibition (Xu et al., 2013). In our study, *IL17A* expression was significantly higher in the SD+T group than in controls. HCD suppressed *IL17* expression, whereas thymol increased it. Previous studies report that obesity, NAFLD, and NASH increase *IL17* expression (Tang et al., 2011; Chackelevicius et al., 2016). As our model represents a non-obese condition, suppression of *IL17* in HCD groups may relate to reduced body weight and adipose tissue. Given IL-17's role in NAFLD progression, the lack of *IL17* increase with thyme oil may indicate a more controlled inflammatory response, potentially limiting progression to NASH.

Flisiak-Jackiewicz et al. (2018) reported elevated serum IL-18 concentrations in patients with NAFLD compared to healthy controls. In the present study, *IL18* expression was higher in the HCD group than in the normal diet group. Furthermore, thyme oil and thymol treatments increased *IL18* gene expression, with a greater increase observed in rabbits receiving thymol. Given that IL-18 is a pro-inflammatory cytokine involved in activating both innate and adaptive immune responses, its upregulation may contribute to hepatic inflammation, fibrosis, and the progression from simple steatosis to NASH. Elevated IL-18 has also been linked to endothelial dysfunction and increased cardiovascular risk, suggesting that its rise in our model may reflect both hepatic and systemic inflammatory activation. Interestingly, our findings on *IL18* expression align with the observed effects on *Tbet* and *IL17*. Thymol markedly upregulated T-bet expression while simultaneously reducing *IL17* expression, suggesting a shift towards a Th1-dominant immune profile and suppression of Th17-mediated responses. Since IL18 is known to synergize with T-bet–driven pathways to enhance Th1 responses, the concurrent increase in IL-18 and T-bet with thymol treatment may represent a coordinated immunomodulatory mechanism. However, while this shift may help restrain IL-17–driven chronic inflammation, it could also potentiate Th1-associated inflammation, which warrants careful consideration in the context of NAFLD progression and cardiovascular risk.

CONCLUSION

In this study, a non-obese rabbit model of hepatic lipidosis, hypercholesterolemia, and hypertriglyceridemia was successfully established using a high-cholesterol diet. This model effectively replicated the hallmark histopathological and immunological changes of non-obese hepatic lipidosis, including extensive microvesicular steatosis, liver cell injury, and pronounced modulation of immune-related gene expression. Thyme oil and thymol did not improve lipid profiles or reduce the severity of fatty liver histopathology; however, both compounds modulated immune gene expression, notably increasing *IL4*, *Gata3*, and in the case of thymol, *Tbet*, while influencing *IL17* and *IL18* expression in a manner suggestive of a shift toward Th1- and Th2-associated responses with reduced Th17 activity. These findings highlight the complex immunomodulatory effects of thyme-derived compounds, which may confer both anti-inflammatory and pro-inflammatory influences depending on the immune pathway involved. While thymol and thyme oil showed potential to modulate hepatic immune responses, their lack of efficacy in improving lipid metabolism or histopathology in this cholesterol-induced model suggests that their therapeutic utility for hepatic lipidosis may be context- and model-dependent. Further studies in diverse hepatic lipidosis models, with varying obesity status, treatment durations, and dosages, are warranted to clarify their role in disease prevention and management.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: VG and GE contributed to the project idea, design and execution of the study. VG and GE contributed to the acquisition of data. VG and GE analysed the data. VG and GE drafted and wrote the manuscript. VG and GE reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

Ethical approval:"Ethics committee approval was received for this study from the Animal Experiments Local Ethics Committee of Erciyes University (Date: 04.03.2020, Number: 20/069). The data, information and documents presented in this article were obtained within the framework of academic and ethical rules."

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